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Soybean Pod Set Enhancement with Synthetic Cytokinin Analogs

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ABSTRACT

The previously reported activity of benzyladenine and selected other cytokinin analogs to increase pod set in soybean (*Glycine max* [L.] Merr.) was further investigated to define the structure-activity relationship and evaluate the effects of the cytokinins on yield parameters. Enhancement of pod set was found to be greatest with N-6 saturated alkyl substituted analogs, and was only weakly associated with activity in a callus growth bioassay. The response of yield parameters to increasing pod load was evaluated by applying various cytokinin analogs having a range of pod set enhancement activity. The increased pod load at the treated nodes was not compensated by a reduction in pod number on the remainder of the plant. However, there was a compensatory decrease in seed size. Overall, a significant trend to greater total seed weight per plant was associated with the increased pod number. Initial evaluations indicated that foliar applications of select cytokinins could temporarily increase pod number. However, the increases in pod number obtained with foliar treatments were too small to be of practical utility and were not maintained to maturity.

In the companion paper (1), we describe the relationship of cytokinin flux to pod set in soybean, and the use of BA as an externally supplied synthetic cytokinin to alter pod set patterns. BA has previously been reported to increase pod number of soybeans under field conditions (4, 10), as well as to increase fruit set in other species (2, 7). The close association between cytokinin flux and pod set in soybean, and the ability to enhance pod set through exogenous applications of BA, make this system especially useful for studying the natural regulation of pod set.

Very few cytokinin analogs have been evaluated for pod set enhancement activity, so little is known regarding the optimum chemical structure to obtain maximal response. A diversity of cytokinin analogs have been reported in the literature (9). The activity of these compounds has usually been tested in tissue or organ bioassays, such as callus growth. Whether the activity in those assays is related to the pod set enhancement activity for the same compounds is not known.

In this paper we report on the results of evaluations comparing the pod set enhancing activity of naturally occurring cytokinins to that of BA. In addition, we characterized the relative activity of a number of synthetic cytokinins, and defined chemical attributes associated with high pod set enhancing activity. Finally, by utilizing several cytokinin analogs which varied in pod set enhancement activity, we report the effects of the cytokinin treatments on yield parameters of treated plants.

MATERIALS AND METHODS

Plant Culture. Soybean (*Glycine max* L. Merr. experimental line IX93-100) plants were grown in 7.6 L pots in a mixture of sand:peat:soil (1:1:2 v:v:v). This genotype produces extended main stem racemes (3–5 cm) on which flowers open individually and sequentially from the base to the tip at a rate of approximately one flower per day. Seeds of IX93-100 were obtained from C. D. Dybing, USDA-ARS, Brookings, SD, via W. A. Brun, University of Minnesota, St. Paul. IX93-100 was selected from crosses made by D. E. Green, Iowa State University, of A71-5558-1 and L61-344, a semideterminate isoline of 'Harosoy.'

Plants were grown in a greenhouse with a 13.5 h photoperiod maintained with supplemental metal halide lamps, which supplied a photosynthetic photon flux density of 300 μE m^{-2} s^{-1} PAR, and temperature was regulated to 28°C day/22°C night. Seeds were inoculated with *Bradyrhizobium japonicum* (Nitragin, Nitragin Co., Milwaukee, WI) at the time of planting. Plants were thinned to one plant per pot at the unifoliate leaf stage (V1 according to Fehr *et al.* [5]). All branches below the first main stem raceme (node 9 or 10) were removed to ensure uniform plant material. Plants were fertilized beginning at the V3 stage with 40 ml of a commercial fertilizer solution containing 16.7 mM NO_3, 41.5 mM NH_4, 36.8 mM urea, 52.9 mM P as orthophosphate, and 76.2 mM K. At 2 week intervals thereafter, each pot was supplied with 150 ml of this nutrient solution. Plants were irrigated with tap water twice daily. Under these conditions the plants had 12 to 14 pod bearing nodes at maturity.

Pod Set Tests. Cytokinin and control treatments were formulated according to the method of Crosby *et al.* (4). Three sequential main-stem racemes in the center of the plant were treated. Racemes were treated when pods (defined as an ovary which had elongated sufficiently to extend past the calyx) were present at 25 to 50% of the proximal floral positions. Pod number at the treated racemes was evaluated at harvest maturity. In the experiment evaluating yield parameters, data were collected for the pod and seed numbers and total seed weights for treated and untreated portions of the plant. Seed weights were measured after seeds had dried to constant weight at 60°C.

Callus Bioassay. The callus bioassay technique used was a modification of the system described by Manos and Goldthwaite (8). Culture dishes were prepared for assay using a liquid medium system with a foam support, similar to that described by Conner and Meredith (3). The culture medium used was a modified Schenk and Hildebrandt (11) medium (pH 5.5), containing 145 mM sucrose, 60 mM glutamine, 1 mM methionine, no antibiotics, and 1 μM naphthaleneacetic acid. Six ml of filter sterilized control medium was placed into each 60 × 15 mm Petri dish. Cytokinin test compounds were dissolved in 25% (v/v) aqueous DMSO

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and filter sterilized prior to being dispensed into the Petri dishes to give the desired 1 μM concentration. This method resulted in a final DMSO concentration of 0.025%, which was found to have no effect on callus growth in preliminary tests. Our initial results identified the 1 μM concentration as optimum for distinguishing relative activity of the cytokinin analogs.

Surface-sterilized soybean seeds were germinated for 5 d at 30°C in darkness under aseptic conditions. The elongated hypocotyls which developed on the germinating seedlings were then aseptically removed and sliced into uniform disks 1 mm in length using a specially designed cutter assembly comprised of 12 razor blades spaced equally in a stainless steel block. Five hypocotyl disks were placed flat onto the filter paper in each dish. Five replications of each treatment were established. Petri dishes were incubated at 30°C for 8 d, after which the tissue pieces were removed and dried at 60°C prior to weighing.

Table I. *Effect of Exogenously Applied Cytokinins on the Number of Pods which Developed on Treated Racemes of IX93-100 Soybean Plants*

Means with the same letter within a column are not significantly different at P = 0.05 according to the Ryan-Elliot-Gabriel-Welsch multiple F-test. Values are means of 5 racemes on each of 5 plants.

Cytokinin Applied	Time After Treatment	
	3 Weeks	6 Weeks
	number of pods per raceme	
Benzylaminopurine	11.4 a	9.3 a
Dihydrozeatin	9.5 b	8.4 ab
Zeatin	8.5 bc	7.7 bc
Dihydrozeatin riboside	8.5 bc	7.2 bcd
Kinetin riboside	6.9 d	6.6 cde
Control	9.0 bc	6.2 def
Isopentenyladenosine	7.4 cd	5.6 ef
Isopentenyladenine	8.0 d	5.4 ef
Zeatin riboside	6.8 d	5.2 f

RESULTS

Activity of Naturally Occurring Cytokinins. When used at a concentration of 2 mM in sprays directed at the raceme, BA resulted in a greater average enhancement of pod set than any of the naturally occurring cytokinins tested (Table I). Zeatin and dihydrozeatin, however, were not significantly less active than BA. None of the other compounds evaluated had any significant enhancement activity, including the other synthetic cytokinin tested, kinetin riboside. The lack of activity for *i*-pentenyladenine and *i*-pentenyladenosine is in keeping with the low relative biological activity of these cytokinins (9).

These results are consistent with similar data reported by Crosby *et al.* (4) for the activity of BA, zeatin, *i*-pentenyladenine, and *i*-pentenyladenosine on Shore soybeans. The relative activity of the free bases is also in good agreement with the relative activity of the same compounds in retarding soybean leaf senescence, as reported by Garrison *et al.* (6). The lack of pod set enhancement activity for the ribosides may be related to the lack of absorption of these highly polar compounds through the external tissues.

Comparison of Pod Set and Callus Activities. In addition to the compounds included in Table I, a large number of synthetic cytokinin analogs were tested at 1 μM in a callus bioassay system to characterize cytokinin-like activity. Many of these compounds were also tested for pod set enhancement activity obtained with a 2 mM direct raceme treatment. Five replicate plates for the callus assay and 15 replicate racemes for the pod set enhancement test were used. The callus assay and pod set enhancement results are summarized in Table II. These two biological activities were compared with an analysis of covariance which separated the compounds into homologous classes dependent on the N-6 and N-9 substitutions. In Table II, the first six compounds were grouped as N-6 saturated alkyl, compounds 7 through 12 as N-6 benzyl, compounds 13 through 16 as N-6 hydroxyalkyl, compounds 17 through 19 as N-6 furfuryl, and compounds 20 and 21 as N-6 unsaturated alkyl. The results of the covariance analysis are summarized in Table III using the N-6 benzyl, N-9 hydrogen group (which includes BA) as the basis of comparison.

Table II. *Pod Set Enhancement and Callus Dry Weight Stimulation Activities of Various Cytokinin Analogs*

Compound Number	N-6 Substituent	N-9 Substituent	Mean Percent Increase in Pod Number	Callus Dry Weight
				mg
1	*n*-Pentyl	Tetrahydropyran	60	4.7
2	*n*-Hexyl	Hydrogen	78	12.0
3	*n*-Hexyl	Ribose	62	19.4
4	*n*-Pentyl	Hydrogen	56	26.4
5	*i*-Pentyl	Ribose	84	32.2
6	*i*-Pentyl	Hydrogen	96	32.4
7	*m*-Fluorobenzyl	Tetrahydropyran	38	4.6
8	Benzyl	Tetrahydropyran	47	29.5
9	*m*-Fluorobenzyl	Ribose	56	31.1
10	Benzyl	Hydrogen	64	33.1
11	Benzyl	Ribose	49	34.3
12	*m*-Fluorobenzyl	Hydrogen	80	34.8
13	4-Hydroxy-3-methylbutyl	Ribose	17	32.3
14	4-Hydroxy-3-methylbuten-2-yl	Ribose	−25	28.3
15	4-Hydroxy-3-methylbuten-2-yl	Hydrogen	28	26.2
16	4-Hydroxy-3-methylbuten-2-yl	Hydrogen	44	31.9
17	Furfuryl	Tetrahydropyran	−5	4.1
18	Furfuryl	Ribose	2	32.6
19	Furfuryl	Hydrogen	15	30.0
20	3-Methylbuten-2-yl	Hydrogen	−22	17.2
21	3-Methylbuten-2-yl	Ribose	−17	16.2

The overall model had R^2 of 0.86 and was significant at the 0.0001 level.

While there was no significant correlation of pod set and callus activities over the entire population of compounds, when the compounds were separated into homologous classes in the analysis the relationship between the two activities was positive and significant at approximately the 0.01 level. The compounds with an N-9 ribose substitution were found to be significantly less active on average in increasing pod set than their respective free bases. By far the most important determinant of pod set enhancement activity was the N-6 substitution. The N-6 saturated alkyl analogs had a mean pod set enhancement 24% greater than the N-6 benzyl class. The N-6 hydroxyalkyls, N-6 furfuryls, and the N-6 unsaturated alkyls had pod set enhancements of 38, 45, and 55% less than the N-6 benzyl class, respectively.

Table III. *Covariance Analysis of Callus and Pod Set Enhancement Activities for Compounds in Table II*

Activity expressed relative to N-6 benzyl N-9 hydrogen analogs.

Substituent Type	Change in Percent Pod Set Enhancement	Probability Level
N-9 riboside	−19	0.021
N-6 saturated alkyl	+24	0.022
N-6 hydroxyalkyl	−38	0.002
N-6 furfuryl	−45	0.001
N-6 unsaturated alkyl	−55	<0.001

Effect of Pod Enhancement on Yield Parameters. Yield parameters were determined on plants treated with the different cytokinin analogs. As the pod number in the treated portion of the plant increased as a result of treatment with the more active compounds, there was no associated change in pod number in the untreated portions of the plant (Fig. 1A). With the lack of compensation for pod number, the total pod number on the plant was found to increase concomitant with the increase in pod number on the treated nodes (Fig. 1B). There was a significant decline in the average seed size associated with the increase in pod number (Fig. 1C). Overall there was a significant increase in total seed weight per plant associated with the increased seed number (Fig. 1D).

Activity of Foliar Treatments. Probably the greatest limitation to applying the pod set enhancement activity in physiological studies or in agronomic practice is that the activity is only obtained through directed sprays on the racemes. It would be especially beneficial to identify compounds which express the pod set enhancement activity when applied to the foliage. To evaluate the potential for foliar application of cytokinins to increase pod set, three compounds which gave substantial enhancement when applied to the racemes were evaluated for their activity when applied to the foliage. The summary in Table IV shows that the response to the raceme application was similar to our previous results. When applied to the foliage, all three compounds gave small but significant increases in the number of pods when evaluated 3 weeks after treatment. However, at

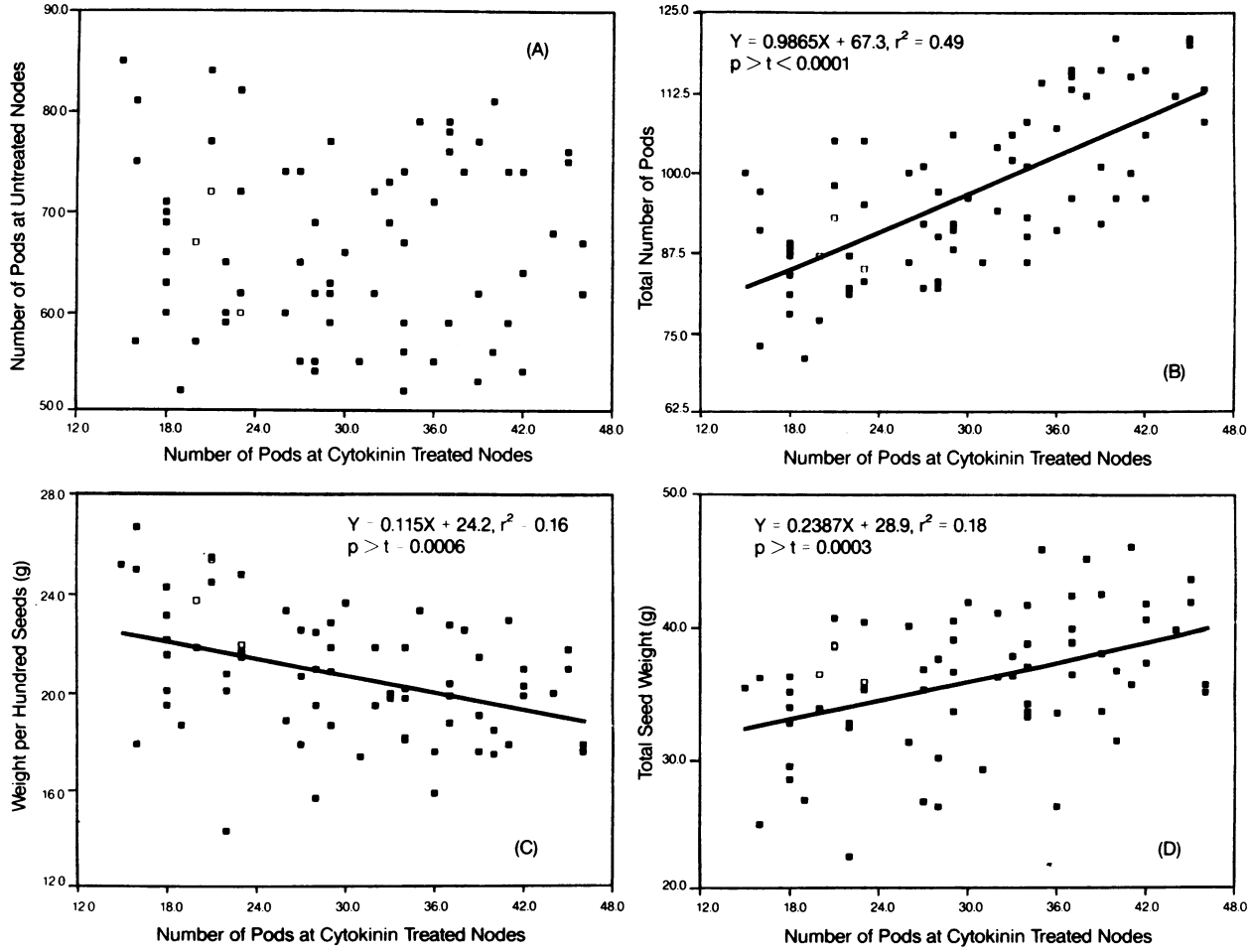

FIG. 1. Effect of cytokinin-induced increases in pod set on: pod set in the untreated portion of the plant (A), total pod set on the plant (B), mean seed size for all seed from the plant (C), and total seed weight per plant (D). Each point represents an individual plant from all cytokinin treated (■) and control (□) plants.

Table IV. *Comparison of the Effect on Pod Set of Cytokinins Applied on the Foliage or Directly on the Racemes*

Means with the same letter within a column are not significantly different at P = 0.05 according to the Ryan-Elliot-Gabriel-Welsh multiple F-test. Means are for three racemes on each of 15 plants.

Cytokinin Applied	Number of Pods per Raceme and Time after Treatment Applied on			
	3 Weeks		6 Weeks	
	Foliage	Raceme	Foliage	Raceme
Control	4.9 b	5.2 b	4.9 a	5.0 b
i-Pentyladenine	5.6 a	6.6 a	5.4 a	5.8 a
n-Hexyladenine	5.6 a	6.2 a	5.3 a	5.8 a
i-Pentyladenine-THP[a]	5.7 a	5.4 b	5.4 a	4.9 b

[a] *i*-Pentyladenine-THP: (N-6-i-pentyl)-(N-9-tetrahydropyranyl)-adenine.

maturity (6 weeks after treatment), the pod number enhancements associated with foliar treatments were not significant. While these increases with foliar treatments were too small to be useful in either physiological studies or agronomic practice, they do indicate the possibility that, given the correct compound, a foliar application of a cytokinin analog may be feasible to obtain a sufficient increase in pod load to result in a measurable change in seed yield.

DISCUSSION

Pod set was enhanced by exogenous applications of cytokinins. Those cytokinin analogs with a saturated alkyl N-6 side chain had superior activity in enhancing pod set. Within any homologous series, the pod set enhancement activity was weakly related to the cytokinin activity of the compound in the callus bioassay. In a practical sense, however, the callus activity was overall a poor indicator of pod set enhancement activity.

Despite a compensatory decline in seed size, the increase in pod number was associated with an increase in seed yield per plant. The increased total seed weight per plant associated with the increasing pod number indicates that the soybean plant is capable of supporting a greater pod load. However, the compensatory decrease in seed size implies that the increased reproductive load placed a demand on the plant that it was not entirely able to supply. Therefore, to obtain significant increases in seed yield will require either a substantial increase in pod number or the identification of mechanisms by which seed size can be maintained despite the increased reproductive load.

That it was only possible to obtain significant enhancement activity by direct application to raceme tissues limits the use of this effect to studies concerning the physiological effects of increased reproductive demand. Some pod set enhancement was obtained with applications of the cytokinin analogs to the foliage. Practical application of the observed activity still requires development of cytokinin analogs capable of expressing the pod set enhancement activity through an agronomically feasible application method.

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